HHS Public Access

Author manuscript

Toxicol In Vitro. Author manuscript; available in PMC 2016 June 01.

Published in final edited form as:

Toxicol In Vitro. 2015 June ; 29(4): 716–721. doi:10.1016/j.tiv.2015.02.002.

Identification of di-2-ethylhexyl terephthalate (DEHTP) metabolites using human liver microsomes for biomonitoring applications

Manori J. Silva[*], **Ella Samandar**, **Antonia M. Calafat**, and **Xiaoyun Ye**

Division of Laboratory Sciences, National Center for Environmental Health, Centers for Disease Control and Prevention, Atlanta, GA 30341, United States

Abstract

Di-2-ethylhexyl terephthalate (DEHTP), a structural isomer of the plasticizer di-2-ethylhexyl phthalate (DEHP), is used in food packaging and medical devices, among other applications, and is a potential replacement for DEHP and other ortho-phthalate plasticizers. Identifying sensitive and specific biomarkers of DEHTP is necessary to assess humans' background exposure to DEHTP. Using mass spectrometry, we investigated the metabolism of DEHTP by human liver microsomes to identify *in vitro* DEHTP metabolites. We unequivocally identified terephthalic acid (TPA) and mono-2-ethylhydroxyhexyl terephthalate (MEHHTP), using authentic standards, and tentatively identified mono-2-ethylhexyl terephthalate (MEHTP) and two other oxidative metabolites of DEHTP: mono-2-ethyloxohexyl terephthalate (MEOHTP), and mono-2-ethyl-5-carboxypentyl terephthalate (MECPTP) from their mass spectrometry fragmentation patterns. We also evaluated the formation of *in vitro* metabolites of DEHP. DEHTP and DEHP produced similar metabolites, but their metabolite profiles differed considerably. DEHTP metabolized to form TPA, a metabolite of several terephthalates, as the major *in vitro* metabolite, followed by MEHTP, MEHHTP, MEOHTP and MECPTP. MEHTP, MEHHTP, MEOHTP and MECPTP, which are specific metabolites of DEHTP, may be suitable biomarkers for assessing exposure to DEHTP. Nonetheless, data on the urinary excretion fraction and temporal stability of these metabolites, among other considerations, are needed to demonstrate their utility as exposure biomarkers.

Keywords

Di-2-ethylhexyl terephthalate; DEHTP; Biomonitoring; Environmental exposure; Oxidative metabolites

[*] Corresponding author at: Centers for Disease Control and Prevention, 4770 Buford Hwy, Mailstop F53, Atlanta, GA 30341, United States. Tel.: +1 (770) 488 7982; fax: +1 (770) 488 0333. zca2@cdc.gov (M.J. Silva)..

Disclaimer

The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the Centers for Disease Control and Prevention. The authors declare they have no competing financial interests.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Transparency Document

The Transparency document associated with this article can be found in the online version.

1. Introduction

Di-2-ethylhexyl terephthalate (DEHTP; Eastman 168™) is considered a safe alternative to its structural isomer, di-2-ethylhexyl phthalate (DEHP), a commonly used plasticizer (Barber, 1994; Barber and Topping, 1995; Gray et al., 2000). For example, animal studies suggested that perinatal exposure to DEHP but not DEHTP altered male sexual differentiation (Gray et al., 2000). Furthermore, there were no adverse effects from DEHTP on reproductive tissue, kidneys, liver hepatocytes, and peroxisomes, which are known targets of DEHP toxicity (Wirnitzer et al., 2011).

DEHTP is used as a plasticizer in flexible polyvinyl chloride, in toys and childcare articles, and in medical devices (Eastman Chemical Company, 2011). DEHTP has also Food Contact Notification clearance from the US Food & Drug Administration (Eastman Chemical Company, 2011) and also complies with the European Commission regulation for use in food contact applications (European Food Safety Authority, 2008).

The usage of DEHTP may be increasing as suggested by a study showing rising levels of DEHTP in dust samples of German households from 1997 to 2009 (Nagorka et al., 2011). However, data on human exposure to DEHTP do not exist. Studies to assess the extent of human exposure to DEHTP at environmental levels require the identification of sensitive and specific exposure biomarkers.

Rats dosed with ^{14}C-DEHTP eliminated most of its radioactivity in feces as unchanged DEHTP and excreted smaller amounts of mono-2-ethylhexyl terephthalate (MEHTP), terephthalic acid (TPA) and other polar metabolites in urine (Barber et al., 1994). However, human metabolites of DEHTP are unknown. *In vitro* studies have been used to identify metabolites of xenobiotic chemicals (Moslemi et al., 1993; Muhitch, 1993; Treadway and Pelkonen, 2006; Zulalian et al., 1993) which can be used as biomarkers of exposure to these chemicals (Silva et al., 2013b).

In the present study, we used mass spectrometry to investigate the metabolism of DEHTP using human liver microsomes and to identify DEHTP exposure biomarkers for human biomonitoring. We also compared the *in vitro* metabolite profiles of DEHP and DEHTP.

2. Experimental

2.1. Reagents and standards

DEHTP, TPA, phthalic acid (PA), and $^{13}C_2$-PA were purchased from Sigma–Aldrich (St. Louis, MO, USA). Mono-2-ethylhexyl phthalate (MEHP) and $^{13}C_4$-MEHP were purchased from Cambridge Isotope laboratories (Andover, MA, USA). Mono-2-ethyl-5-carboxypentyl phthalate (MECPP), a specific isomer of mono-2-ethylhydroxyhexyl terephthalate (MEHHTP), namely mono-2-ethyl-5-hydroxyhexyl terephthalate, and $^{13}C_6$-MECPP were purchased from CanSyn (Ontario, Canada). Mono-2-ethyl-5-hydroxyhexyl phthalate (MEHHP), mono-2-ethyl-5-oxohexyl phthalate (MEOHP), and their deuterated analogs were purchased from ADM (Teltow, Germany). The stock standard solutions were prepared in acetonitrile and the calibration standards were prepared in 10% acetonitrile in water (Silva

et al., 2007). All reagents, solvents and standard materials were used without further purification.

2.2. In vitro metabolism

In a 30 mL Qorpak™ Clear Wide Mouth French square bottle (Fisher Scientific, Pittsburg, PA, USA), a DEHTP standard solution (100 lL; 769.6 μg/mL) was mixed with pH 7.4 phosphate buffer (0.1 M, 8 mL), water (1 mL), NADPH solution (A) (500 μL, BD Gentest™), NADPH solution (B) (100 μL BD Gentest™), and female human liver microsome homogenates (200 μL, BD Gentest™, Woburn, MA, USA). The bottle was capped and the contents were gently mixed and placed in an incubator (Fisher Scientific, Hampton, NH, USA) at 37 °C for 5 h. Aliquots of microsomal suspension (1 mL) were transferred into microcentrifuge tubes and vortex mixed, before being centrifuged at 12,500 rpm for 20 min on an Avanti high performance centrifuge (Beckman Coulter Inc., Brea, CA, USA). The supernatant was transferred into autosampler vials for analysis. The above procedure was repeated without DEHTP, but with water (100 μL) for the preparation of the control samples.

For the time course study, DEHTP or DEHP standard solution (1000 μL; 250 μg/mL) was dried down to 100 μL under a stream of nitrogen and was mixed with pH 7.4 phosphate buffer (0.1 M, 7 mL), water (1 mL), NADPH solution (A) (600 μL), NADPH solution (B) (100 μL), and male human liver microsome homogenates (200 μL) in a 30 mL Qorpak™ Clear Wide Mouth French square bottle. The bottle was capped and the contents were gently mixed and placed in an incubator at 37 °C. At several time intervals between time 0 and 27 h, 100 μL aliquots ($N = 3$) of microsomal suspension were withdrawn into microcentrifuge tubes containing acetonitrile (200 μL) to quench the enzymes and 100 μL of an internal standard solution (Silva et al., 2007) prepared with $^{13}C_2$-PA, $^{13}C_4$-MEHP, D_4-MEOHP, D_4-MEHHP, and D_4-MECPP in 10% aqueous acetonitrile. The contents in the microcentrifuge tubes were vortex mixed, and the tubes were stored at −70 °C. After the last sample was withdrawn, all samples were thawed at once and centrifuged at 12,500 rpm for 20 min. The supernatants were transferred into autosampler vials for analysis.

2.3. Identification of DEHTP metabolites

The HPLC gradient for separation of DEHTP metabolites and the on-line SPE procedure were adapted from previously published methods (Silva et al., 2007, 2013a). Briefly, metabolites in the supernatant of the human liver microsomal homogenate (500 μL) obtained after incubating with DEHTP for 5 h were extracted using on-line SPE on a Chromolith RP-18 pre-column (Merck KGaA, Darmstadt, Germany), resolved on a Betasil phenyl HPLC column (3 lM, 2.1 mm × 25 mm, ThermoFisher Scientific, San Jose, CA, USA) using a water/acetonitrile gradient, and detected by mass spectrometry on a TSQ Vantage AM triple quadrupole mass spectrometer (ThermoFinnigan, San Jose, CA, USA). All ions on Q1 were scanned from $m/z = 125$ to $m/z = 325$ in electrospray ionization (ESI)-negative ion mode. ESI Q1 full scan produced multiple peaks. The fragmentation patterns of the major peaks were analyzed to identify potential DEHTP metabolites (Table 1). Metabolites unique to DEHTP (Fig. 1) were identified by comparing the mass transitions of the peaks resulting from human liver microsome incubate with DEHTP to those of human liver microsome

homogenate without DEHTP. Product ion scans were performed for major peaks, namely *m*/*z* = 165, 277, 291, 293, and 307.

2.4. Chromatographic separation and mass spectrometric detection of DEHP and DEHTP metabolites

DEHP and DEHTP metabolites were chromatographically separated and analyzed by mass spectrometry in negative ion, multiple reaction monitoring mode (Fig. 2) using a previously published approach (Silva et al., 2007, 2013a). DEHTP metabolites produced fragments with mass spectrometric transitions similar to those of the metabolites of DEHP (Figs. 3 and 4). Therefore, to the supernatant of a human liver microsomal homogenate (500 μL) obtained after incubating 5 h with DEHTP, we added a solution containing five DEHP metabolites, namely PA, MEHHP, MEOHP, MECPP, and MEHP (100 μL, 50 ng/mL) to evaluate chromatographic separation of DEHTP metabolites in the presence of DEHP metabolites.

2.5. Comparison of major metabolites of DEHP and DEHTP

Product ion scans of the DEHTP metabolites identified tentatively (*m*/*z* = 165/121, 277/233, 291/247, 293/121, and 307/121) in the human liver microsome homogenate after incubating with DEHTP were performed by injecting the supernatant of the microsomal homogenate (100 μL) to a Vantage AM triple quadrupole mass spectrometer after the HPLC separation described above. The procedure was repeated with a standard solution mixture (1 μg/mL) containing PA (*m*/*z* = 165/121), and the DEHP metabolites MEHP (*m*/*z* = 277/233), MEHHP (*m*/*z* = 293/121), MEOHP (*m*/ *z* = 291/247), and MECPP (*m*/*z* = 307/121). The comparison mass spectra are presented in Figs. 3 and 4.

2.6. Quantification of DEHTP and DEHP metabolites

The target metabolites from human liver microsomes homogenates incubated with DEHTP or DEHP for up to 27 h were measured by using on-line SPE–HPLC–tandem mass spectrometry as previously described (Silva et al., 2007, 2013a). The mobile phase contained 0.1% acetic acid in water and 0.1% acetic acid in acetonitrile. We did not attempt to characterize the individual isomers of MEHHTP or other DEHTP metabolites. PA, MEHP, MEHHP, MEHHTP, MEOHP, MECPP, and TPA were quantified using authentic standards. MEOHTP, and mono-2-ethyl-5-carboxypentyl terephthalate (MECPTP) were quantified using their corresponding isomeric DEHP metabolites (Table 1). The limits of detection (LODs) were 0.5 ng/mL (PA, TPA, MEHHTP, MEHP, MECPP) and 0.2 ng/mL (MEHHP, MEOHP).

3. Results and discussion

Metabolism of DEHTP using human liver microsomes (Fig. 1) formed analogous metabolites to those of DEHP. Full scan analysis in negative ion mode from *m*/*z* = 125 to *m*/*z* = 325 of the human liver microsomes supernatant after 5 h incubation with DEHTP resulted in multiple peaks (Fig. 2). Metabolites of DEHP, added post-incubation to the supernatant of the DEHTP microsomal homogenate, eluted earlier than their analogous DEHTP metabolites (Fig. 2). For DEHTP, we unambiguously identified TPA (*m*/*z* = 165,

retention time [RT] = 5.5 min) and three MEHHTP isomers (*m*/*z* = 293, RT = 15.5, 16.9 and 18.6 min) using authentic standards, and tentatively identified MECPTP (*m*/*z* = 307, RT = 16.7 min), MEOHTP (*m*/*z* = 291, RT = 19.9 min), and MEHTP (*m*/*z* = 277, RT = 25.7 min) using their mass spectrometric fragmentation patterns (Figs. 2–4). Isomers of MEHHTP with similar fragmentation patterns produced multiple chromatographic peaks between 15.5 and 18.6 min (Fig. 2), and the fragmentation patterns of these isomers matched well with the fragmentation of the MEHHTP authentic standard (Fig. 4). The potential for multiple sites of oxidation also exists for MEOHTP, but MEOHTP eluted as a single broad peak at 19.9 min. We could not determine conclusively whether multiple isomers of MEOHTP co-eluted or the *in vitro* metabolism of DEHTP produced only one isomer of MEOHTP.

DEHTP and DEHP metabolites displayed different fragmentation patterns. The mass spectra of the hydrolytic metabolites TPA and PA, and of MEHTP and MEHP are presented in Fig. 3, whereas those of the oxidative metabolites MEHHP and MEHHTP, MECPP and MECPTP, and MEOHP and MEOHTP are presented in Fig. 4. The major *m*/*z* transition for TPA, MEHHTP, and MECPTP was *m*/*z* = 121 $\left(C_7H_5O_2^-\right)$ at 25 eV collision energy. Under similar conditions, the most abundant fragments for MEHTP (Fig. 3B) and for MEOHTP (Fig. 4C) were *m*/*z* = 233 $[(M\text{-}1)\text{-}CO_2]^-$ and *m*/*z* = 247 $[(M\text{-}1)\text{-}CO_2]^-$, respectively. DEHP metabolites, MEHHP and MEOHP also produced *m*/*z* 121 as their major fragment (Fig. 4). The major fragments of the other DEHP metabolites were *m*/*z* = 77 (PA), *m*/*z* = 134 (MEHP), and *m*/ *z* = 159 (MECPP).

We also evaluated the *in vitro* metabolism of DEHP and DEHTP for up to 27 h (Fig. 5) and noted that the metabolic profile of DEHTP and DEHP differed significantly (Table 2). DEHTP formed MEHTP, MEOHTP, MEHHTP, MECPTP, and TPA which are analogous to the DEHP metabolites MEHP, MEOHP, MEHHP, MECPP, and PA, respectively. TPA was the major metabolite of DEHTP, whereas DEHP mainly hydrolyzed to MEHP, which further metabolized to MEHHP, MEOHP, MECPP, and PA (Table 2). MECPTP was produced only as a minor *in vitro* metabolite of DEHTP. Interestingly, the *in vitro* metabolism of DEHP produced MECPP as a minor metabolite, but MECPP is one of the major DEHP urinary metabolites in humans (Koch et al., 2003; Silva et al., 2007). Similarly, the fraction of DEHTP excreted as MECPTP and other oxidative metabolites may be higher in vivo than *in vitro*, thus warranting further investigations.

In summary, using human liver microsomes, we unequivocally identified TPA as the major *in vitro* metabolite of DEHTP and MEHHTP as one specific metabolite of DEHTP. We also tentatively identified three unique metabolites of DEHTP, specifically MEHTP, MEOHTP, and MECPTP. TPA can be formed by other terephthalates (e.g., di-methyl terephthalate) and, therefore, is not a specific biomarker of exposure to DEHTP. In contrast, MEHTP, MEOHTP, MEHHTP, and MECPTP may serve as specific exposure biomarkers of DEHTP. Nonetheless, additional considerations, such as adequate collection protocols, handling and storage of the samples, and data on the urinary excretion fraction and temporal stability of these metabolites in urine, are needed to demonstrate the utility of these biomarkers for exposure or risk assessment purposes.

Acknowledgements

This work was supported by the Centers for Disease Control and Prevention, U.S. Department of Health and Human Services.

References

Barber ED. Generic toxicology testing of di(2-ethylhexyl) terephthalate. Environ. Mol. Mutagen. 1994; 23:228–233. [PubMed: 8162897]

Barber ED, Topping DC. Subchronic 90-day oral toxicology of di(2-ethylhexyl) terephthalate in the rat. Food Chem. Toxicol. 1995; 33:971–978. [PubMed: 7590545]

Barber ED, Fox JA, Giordano CJ. Hydrolysis, absorption and metabolism of di(2-ethylhexyl) terephthalate in the rat. Xenobiotica. 1994; 24:441–450. [PubMed: 8079503]

Eastman Chemical Company. Eastman 168™ Non-Phthalate Plasticizer Receives Additional U.S. FDA Food Contact Clearance. 2011 <http://www.eastman.com/Company/News_Center/2011/Pages/Eastman168_Non_Phthalate_Plasticizer_Receives_Additional_USFDA_Food_Contact_Clearance.aspx>.

European Food Safety Authority. Opinion of the Scientific Panel on Food Additives, Flavourings, Processing Aids and Materials in Contact with Food (AFC) on a Request Related to a 18th List of Substances for Food Contact Materials. 2008 <http://www.efsa.europa.eu/en/scdocs/doc/628.pdf>.

Gray LE, Ostby J, Furr J, Price M, Veeramachaneni DNR, Parks L. Perinatal exposure to the phthalates DEHP, BBP, and DINP, but not DEP, DMP, or DOTP, alters sexual differentiation of the male rat. Toxicol. Sci. 2000; 58:350–365. [PubMed: 11099647]

Koch HM, Rossbach B, Drexler H, Angerer J. Internal exposure of the general population to DEHP and other phthalates – determination of secondary and primary phthalate monoester metabolites in urine. Environ. Res. 2003; 93:177–185. [PubMed: 12963402]

Moslemi S, Dintinger T, Dehennin L, Silberzahn P, Gaillard JL. Different in vitro metabolism of 7 alpha-methyl-19-nortestosterone by human and equine aromatases. Eur. J. Biochem. 1993; 214:569–576. [PubMed: 8513806]

Muhitch MJ. In vitro metabolism of L-aspartate by maize kernels. Phytochemistry. 1993; 32:1125–1130.

Nagorka R, Conrad A, Scheller C, Sussenbach B, Moriske HJ. Diisononyl 1,2-cyclohexanedicarboxylic acid (DINCH) and Di(2-ethylhexyl) terephthalate (DEHT) in indoor dust samples: concentration and analytical problems. Int. J. Hyg. Environ. Health. 2011; 214:28–37.

Silva MJ, Samandar E, Preau JL, Reidy JA, Needham LL, Calafat AM. Quantification of 22 phthalate metabolites in human urine. J. Chromatogr. B. 2007; 860:106–112.

Silva MJ, Jia T, Samandar E, Preau JL, Calafat AM. Environmental exposure to the plasticizer 1,2-cyclohexane dicarboxylic acid, diisononyl ester (DINCH) in US adults (2000–2012). Environ. Res. 2013a; 126:159–163. [PubMed: 23777640]

Silva MJ, Samandar E, Ye X, Calafat AM. In vitro metabolites of di-2-ethylhexyl adipate (DEHA) as biomarkers of exposure in human biomonitoring applications. Chem. Res. Toxicol. 2013b; 26:1498–1502. [PubMed: 24016063]

Treadway A, Pelkonen O. In vitro screening of drug metabolism during drug development: an interview with Olavi Pelkonen. Expert Opin. Drug Discov. 2006; 1:15–17. [PubMed: 23506029]

Wirnitzer U, Rickenbacher U, Katerkamp A, Schachtrupp A. Systemic toxicity of di-2-ethylhexyl terephthalate (DEHT) in rodents following four weeks of intravenous exposure. Toxicol. Lett. 2011; 205:8–14. [PubMed: 21616130]

Zulalian J, Stout SJ, Dacunha AR, Rajan S. Comparative invitro metabolism of moxidectin in cattle and rat. Abstr. Papers Am. Chem. Soc. 1993; 205:73–AGRO.

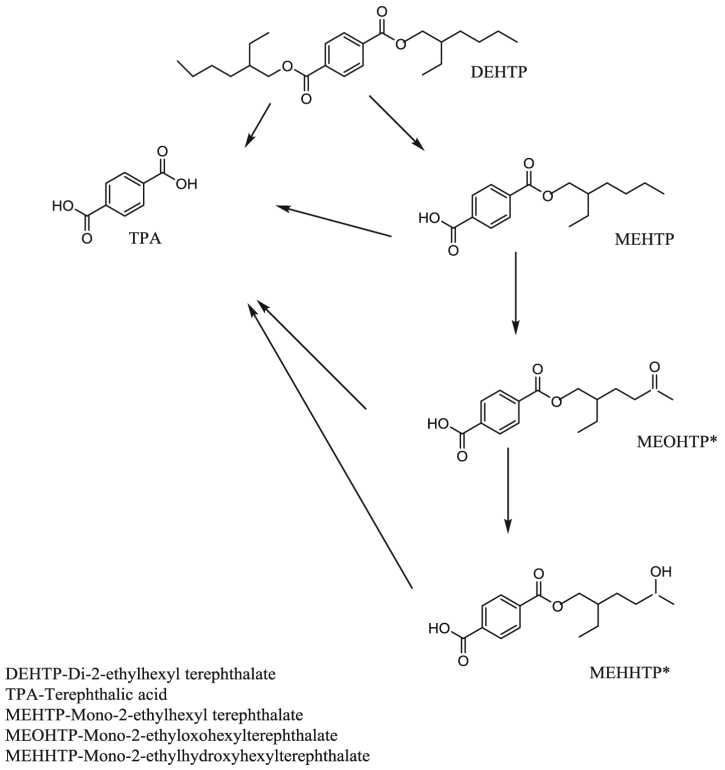

Fig. 1.

Metabolic products tentatively identified in the supernatant of the human liver microsome suspension after incubating with di-2-ethylhexyl terephthalate for 5 h.

*The structures shown for MEOHTP and MEHHTP are for one isomer only.

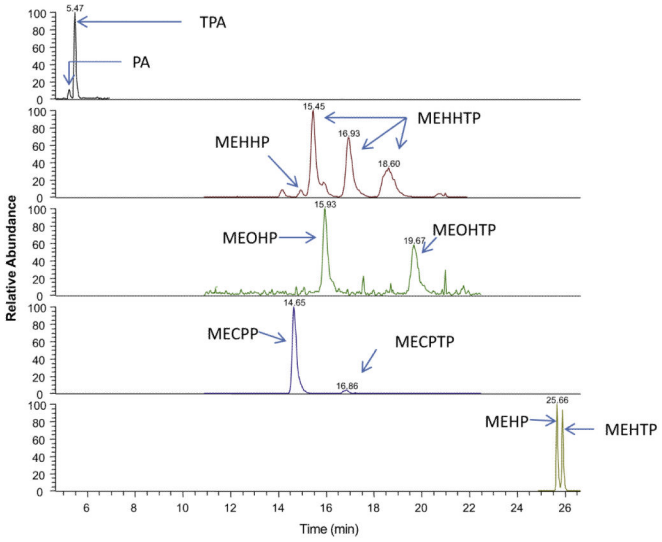

Fig. 2.

Chromatographic separation of di-2-ethylhexyl phthalate and di-2-ethylhexyl terephthalate metabolites detected in the supernatant of the human liver microsomes suspension of DEHTP after 5 h incubation at 37 °C and spiked with DEHP metabolites.

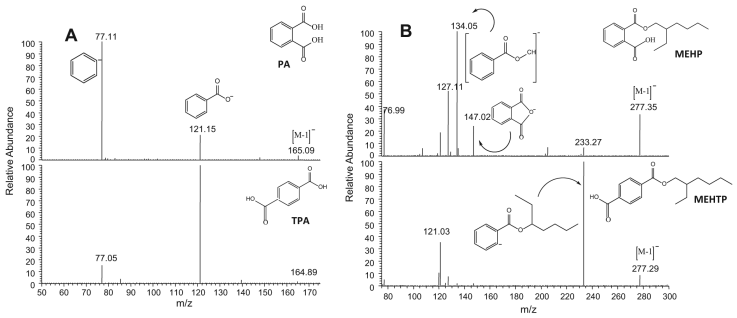

Fig. 3.

Comparison of mass spectrometric fragmentation of hydrolytic metabolites of DEHTP and DEHP: TPA and PA (A), MEHTP and MEHP (B).

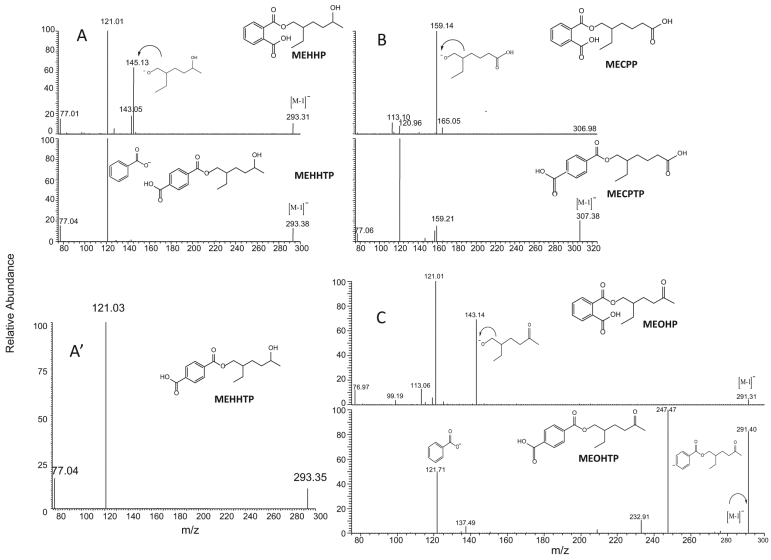

Fig. 4.

Mass spectrometric fragmentation of oxidative metabolites of DEHTP. MEHHTP and MEHHP (A), MEHHTP standard (A′), MECPTP and MECPP (B), and MEOHP and MEOHTP (C).

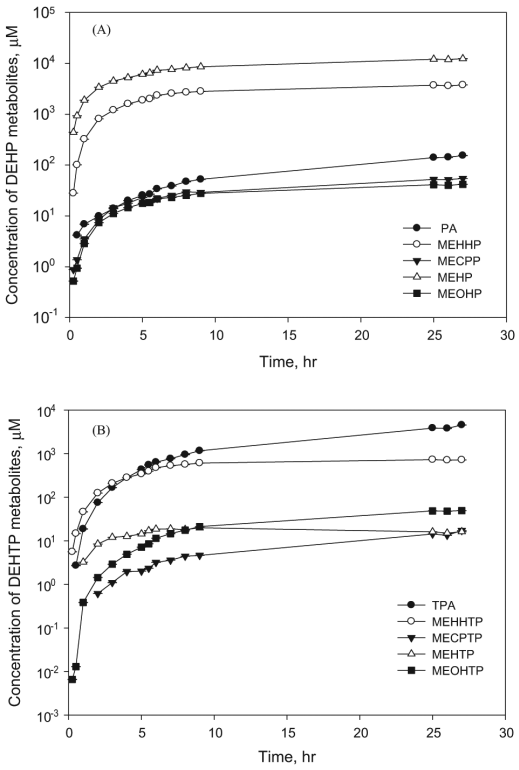

Fig. 5.

In vitro metabolism of DEHP (A) and DEHTP (B) with human liver microsomes. Error bars represent standard deviation. $N = 3$, TPA, MECPTP, MEHTP and MEOHTP were quantified using analogous phthalate metabolites.

Table 1

Mass spectrometric parameters for measuring the metabolites of di-2-ethylhexyl terephthalate (DEHTP).

DEHTP metabolite[a]	m/z	
	Precursor	Product
Terephthalic acid (TPA)	165	121
Mono-2-ethylhexyl terephthalate (MEHTP)	277	233
Mono-2-ethylhydroxyhexyl terephthalate (MEHHTP)	293	121
Mono-2-ethyloxohexyl terephthalate (MEOHTP)	291	247
Mono-2-ethylcarboxypentyl terephthalate (MECPTP)	307	121

[a] Collision energy was 25 eV.

Table 2

Concentration of DEHTP and DEHP metabolites (N = 3) after incubating 640 nmoles of DEHTP and DEHP for 26 h with human liver microsomes at 37 °C.

DEHTP metabolite	Concentration μM mean ± SD	DEHP metabolite	Concentration μM mean ± SD
TPA	27.2 ± 1.17	PA	0.93 ± 0.02
MEHTP	0.06 ± 0.003	MEHP	44.44 ± 1.76
MEHHTP	2.43 ± 0.10	MEHHP	12.84 ± 0.58
MEOHTP	0.17 ± 0.01	MEOHP	0.14 ± 0.01
MECPTP	0.06 ± 0.01	MECPP	0.18 ± 0.01

Terephthalic acid (TPA), mono-2-ethyloxohexyl terephthalate (MEOHTP), mono-2-ethylhydroxyhexyl terephthalate (MEHHTP), mono-2-ethylhexyl terephthalate (MEHTP), mono-2-ethylcarboxypentyl terephthalate (MECPTP), Phthalic acid (PA), mono-2-ethyl-5-oxohexylphthalate (MEOHP), mono-2-ethyl-5-hydroxyhexyl phthalate (MEHHP), mono-2-ethyl-5-carboxypentyl phthalate (MECPP), mono-2-ethylhexyl phthalate (MEHP).